EXHIBIT 4

16

Adult diuresis, excretion and defaecation

This chapter is concerned with the processes that rid the adult body of unwanted substances and ensure homeostasis of the haemolymph under conditions of water and salt loading. The diuretic and excretory processes of adult mosquitoes, like those of the larvae, are variants of the mechanisms that have evolved in insects in general and which we shall briefly review. Insects have a two-part excretory system in which an initial secretion of the Malpighian tubules (tubular fluid), which is isosmotic with the haemolymph, is modified in the rectum before being expelled as urine. Fluid secretion by the Malpighian tubules is driven by the active transport of potassium ions across the epithelium into the tubule lumen. Chloride ions follow passively, and potassium chloride is normally the predominant solute in the tubular fluid. The concentrations of other solutes, including sodium, sugars and amino acids, are low relative to their concentrations in the haemolymph. The tubular fluid enters the pylorus from where some may be moved forwards for resorption in the midgut. The remainder is moved posteriorly to the rectum where it is modified by selective absorption or secretion of water, ions and metabolites, producing urine which may be hyper- or hypo-osmotic to the haemolymph. The volume of fluid excreted is determined by endocrine control of both Malpighian tubule secretion and rectal absorption, different hormones inducing diuretic and antidiuretic activity (Spring, 1990).

Discharge of excretory products may give the impression of two distinct activities, the rapid production of urine containing principally water and salts (diuresis) and the voiding of semi-solid nitrogenous wastes, but these two activities involve related or interactive physiological processes. Through the capacity of the hindgut to resorb water, excretion can be accomplished with a minimum of water loss, permitting water conservation when required. Equally, the Malpighian tubule–hindgut system permits diuresis on occasions of water load and regulates the volume and composition of the extracellular fluid at all times. Defaecation of undigested food residues and other discarded products of the midgut provides a third eliminatory process.

The disposal of waste or surplus substances by adult mosquitoes occurs most conspicuously after emergence and after each blood meal, and nectar feeding also is followed by the disposal of water. After emergence the teneral mosquito voids waste substances that accumulated during metamorphosis and discharges water and salts to reduce its haemolymph volume, which is still extensive like that of the developmental stages, to the lower volume characteristic of the adult stage. Once this fluid has been discharged the remaining body water is carefully conserved.

The large volumes of blood ingested by female mosquitoes not only make flight difficult but also produce water- and salt-loads which threaten the homeostasis of the haemolymph. The excretory system has the capacity to discharge over 40% of the water and sodium contained in the ingested blood plasma within one hour of feeding. During the first day after feeding deamination of amino acids produces excess nitrogen which is voided in the form of low molecular weight nitrogenous metabolites. Defaecation occurs later.

Authors have not been mutually consistent in the use of terms associated with excretion and

diuresis, so it is necessary to define these terms as they are used here.

1. Tubular fluid – the unmodified secretion of the Malpighian tubules.
2. Urine – the completed excretory fluid as discharged to the exterior.
3. Excreta – nitrogenous waste including uric acid, urea and ammonium ion, usually discharged in a semi-solid state but sometimes in the urine.
4. Faeces – undigested food residues and other discarded products of the midgut.
5. Meconium – nitrogenous and other waste products remaining in the gut from the period of metamorphosis.

The term 'excretion' is used here in two senses: broadly, for all eliminatory processes apart from defaecation, and more narrowly for the elimination of nitrogenous compounds.

16.1 DIURESIS AFTER EMERGENCE AND AFTER FEEDING

Insect epithelia that are adapted for the passage of water and ions characteristically have a microvillate apical cell membrane, a deeply infolded basolateral cell membrane, and numerous mitochondria which are associated with both the microvilli and the basal labyrinth. Such an ultrastructure is found in the midgut, the Malpighian tubules, the anterior intestine and the rectal papillae of adult mosquitoes (Sections 13.2, 13.3). At metamorphosis the rectum of the larval mosquito is anatomically transformed to that of the adult while the Malpighian tubules pass to the adult without cell loss or visible change. However, the secretory abilities of the adult tubules differ from those of the larval tubules.

The high osmotic permeability of the Malpighian tubule cell membranes possibly allows solute and water flows to be coupled by osmosis. Thus the movement of water from the haemolymph and across the tubules may be a passive response to osmotic gradients produced in the cells and in the lumen by the active transport of potassium ions which is known to occur. The tubules secrete a potassium-rich fluid at a rate that is sensitive to the potassium concentration of the medium. This system is undoubtedly supplemented by other mechanisms of active transport. There is evidence also of a slow paracellular movement of larger molecules and charged molecules through the intercellular junctions (O'Donnell and Maddrell, 1983; O'Donnell *et al.*, 1984).

Bloodfed mosquitoes need to rid themselves of the high sodium load gained from the ingested plasma and the potassium gained in the blood cells. Adult mosquitoes differ from the majority of insects and from their own larvae in that, in the absence of secretagogues, their Malpighian tubules actively transport both sodium and potassium at similar rates. Under conditions of rapid diuresis the excretory mechanism changes to one based on sodium secretion.

Diuretic hormone activity has been demonstrated in a number of insects, and there is evidence that diuretic hormones are peptides originating in the central nervous system. Mosquitoes release a diuretic hormone during blood feeding. This is thought to stimulate the Malpighian tubule cells to produce cAMP which acts as a second messenger, inducing further changes which produce fluid flow across the tubular epithelium.

The phenomenon of diuresis in bloodfed mosquitoes resembles that in another blood-sucking insect, *Rhodnius prolixus*. The fluid secretion rates of *Rhodnius* Malpighian tubules are said to be the highest reported for any animal (Florey, 1982), but the peak rates of urine flow exhibited by mosquitoes imply similar tubular secretion rates. Detectable amounts of diuretic hormone are found in the haemolymph of individuals of *Rhodnius* that have been imbibing blood for as little as 15 s (Maddrell and Gardiner, 1976). The speedy diuretic response of mosquitoes to blood feeding suggests that hormone release may be equally rapid in them.

Most studies of diuresis in adult mosquitoes have been on *Aedes aegypti*; in the following

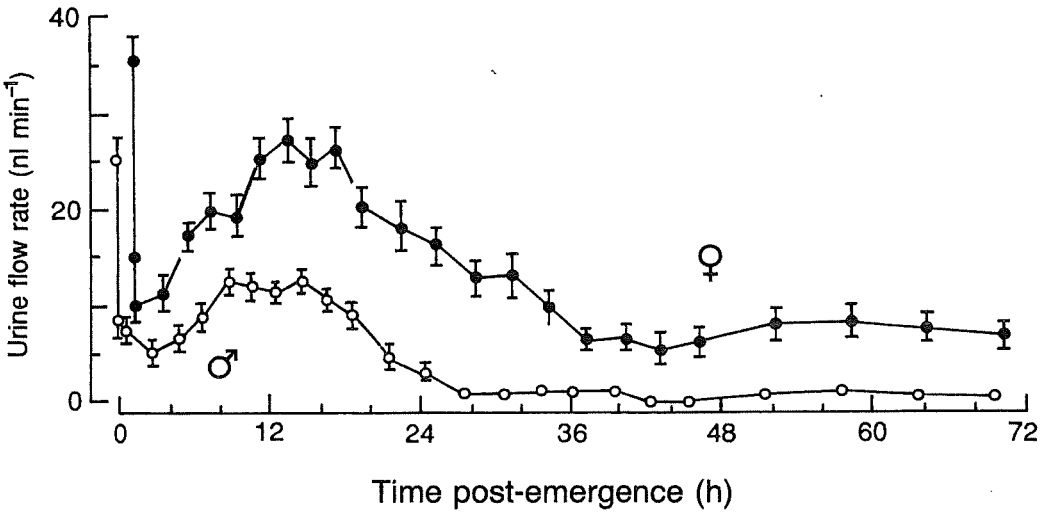

Figure 16.1 Mean rates of urine flow in unfed male and female *Aedes aegypti* during the first three days of adult life. The data points for males and females are shifted slightly to the left and right respectively. (From Gillett, 1983a.)

sections, where no species is named, *Ae aegypti* is the species concerned.

16.1.1 Diuresis after emergence

The teneral mosquito, newly emerged from its pupal skin, is disadvantaged in flight by the weight of haemolymph carried over from its juvenile stages, and discharge of droplets of clear fluid from the anus is the first observable action it performs. The droplets vary in diameter, and an individual may expel a few large drops or many small drops during equal time periods. By counting numbers of droplets and classifying each as large, medium or small (mean measured volumes 34, 25 and 13 nl respectively), it was possible to determine rates of urine production (DeGuire and Fraenkel, 1973; Jones and Brandt, 1981; Gillett, 1982b).

Most *Aedes aegypti* ejected a droplet just before or at the moment the abdomen was freed from the pupal cuticle, and immediately afterwards they discharged urine at a high rate, females at >40 nl min^{-1} and males >25 nl min^{-1}. However, this post-emergence burst of urine flow was short-lived and declined sharply within 20 min (Figure 16.1). From about 3–4 h post-emergence the rate of urine flow started to increase again. In females it increased slowly to a maximum >25 nl min^{-1} by 12–14 h post-emergence, and declined from 15 to 16 h onwards. In males urine flow restarted at the same time but ended earlier; further, the flow rates were lower. After completion of the 'day-1-peak' the rate of urine flow stabilized at 6.9 ± 0.5 nl min^{-1} in unfed females and at 0.6 ± 0.1 nl min^{-1} in unfed males (Gillett, 1983a).

Male and female *Anopheles gambiae* exhibited a post-eclosion burst of diuresis, which peaked between 1 and 5 min after the adult left the pupal skin. In most individuals the burst ended within 12 min, by which time the abdomen had lost its distended appearance (Goma, 1964).

Decapitation within 1 min of leaving the pupal cuticle reduced or eliminated the post-emergence burst of diuresis in *Ae aegypti* (Jones and Brandt, 1981). Decapitation at any time from 1 to 14 h post-emergence greatly reduced urine flow in the day-1-peak, the flow rate varying slightly with time of decapitation (Gillett, 1983a).

16.1.2 Diuresis after feeding

Meals of nectar and other plant juices are stored in the crop and their water content is discharged slowly. Sugar solution imbibed by *Ae aegypti* was first seen in the midgut about 30 minutes after the crop had filled, and females that drank 10% sugar solution discharged no fluid or at most a

few droplets during the first 30 min after feeding (Jones and Madhukar, 1976; Jones and Brandt, 1981). Sugarfed females of *Ae taeniorhynchus* lost weight at a slow constant rate which averaged 2.2–2.7 μg min^{-1} during the first 50 min and 1.7 μg min^{-1} between 50 and 90 min after feeding (Nayar and Bradley, 1987). This was probably greater than their transpiration rate since the transpiration rate of female *Ae aegypti* with sealed anus was 0.55 μg min^{-1} (Stobbart, 1977).

Like many mosquitoes, females of *Aedes aegypti* that feed to repletion ingest more than their own weight in blood. Diuresis often commences some 50–75 s after the start of feeding, and up to ten or more droplets of urine may be passed before feeding has ended. Extrusion of a droplet of urine, which takes a fraction of a second, involves a tilting upwards and telescoping of the terminal abdominal segments, rectal peristalsis, splaying of the cerci, and opening of the anus. The anus closes suddenly upon completion of the rectal peristalsis, segments 7 and 8 move slightly, and the droplet is forcefully ejected, often over 5–10 mm. Strains of *Aedes aegypti* differ in the rate of production and uniformity of size of the excretory droplets. The fluid discharged by bloodfed *Ae aegypti* must have passed through the Malpighian tubules since trypan blue or cerium–144, if present in the ingested blood, do not appear in the urine. The first one or two drops of urine may be cloudy, and these contain uric acid; the remainder are clear and free of uric acid but contain ninhydrin-positive substances (Boorman, 1960; Redington and Hockmeyer, 1976; Stobbart, 1977; Jones and Brandt, 1981; Mellink *et al.*, 1982).

Rates of urine production by bloodfed mosquitoes have been measured by counting the number of droplets discharged. Experiments with one strain of *Ae aegypti* showed droplet volume to be consistently within the range 10–12 nl, so urine production could be calculated directly from droplet numbers. Urine production passed through three phases which differed in rate of flow: (i) a **peak phase** of very rapid flow in the first few minutes after the blood meal, (ii) a **post-peak phase** of declining flow rate, and (iii) a **late phase** when the flow rate was slightly above the control level. In one female the peak phase of diuresis lasted a little over ten minutes, with the flow rate reaching a maximum of 54 nl min^{-1} six minutes after the start of blood feeding. The post-peak phase lasted about 60 min, and by the end of it the urine flow rate had fallen to about 2–6% of that at the peak (Figure 16.2, Table 16.1B) (Williams *et al.*, 1983; Petzel *et al.*, 1987).

Urine production has also been recorded by weighing females at short intervals after the blood meal. One gravimetric measurement of urine production by *Ae aegypti* revealed the same three phases of urine production, but with the peak phase lasting 25–35 min. By some 60–120 min post-bloodmeal the weight losses could be accounted for by transpiration (Stobbart, 1977). Whenever the droplet method has been used it has indicated a short initial peak (Boorman, 1960; Jones and Brandt, 1981; Williams *et al.*, 1983), but strain differences cannot be ruled out as the cause of this difference.

Comparisons of the osmolality and the sodium, potassium and chloride concentrations of *Ae aegypti* haemolymph with those of human plasma (Table 16.1A) provide a measure of the immediate osmotic and ionic loads imposed on the

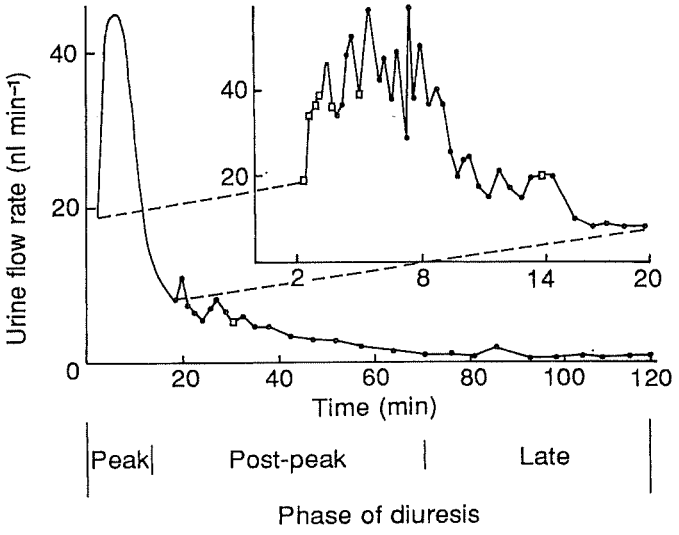

Figure 16.2 Urine flow rate in a single female *Aedes aegypti* during the first two hours after feeding to repletion on human blood, the blood meal commencing at time 0. Inset, an expansion of the time scale for the peak phase of the diuresis. (From Williams *et al.*, 1983.)

Table 16.1 Composition of the haemolymph and urine of *Aedes aegypti* and of human blood plasma. (From Williams *et al.*, 1983.)

(A) Composition of haemolymph from non-bloodfed mosquitoes (means ± s.e.) and of clinical ranges of human plasma.

Parameter	*Haemolymph*	*Human plasma*
Osmolality (mosmol kg^{-1})	354 ± 14	280–295
$[Na^+]$ (mM)	96 ± 7	136–146
$[K^+]$ (mM)	6.5 ± 1.0	3.5–5.5
$[Cl^-]$ (mM)	135 ± 19	96–106

(B) Urine flow rate and composition at selected times during diuresis following a meal of human blood by *Aedes aegypti* (means ± s.e.).

Parameter	*Phase of diuresis*		
	Peak	*Early post-peak*	*Late post-peak*
Time post-bloodmeal (min)	6	26	61
Flow rate (nl min^{-1})	54.4 ± 5.2	11.1 ± 4.3	2.6 ± 0.5
Osmolality (mosmol kg^{-1})	309 ± 7	217 ± 16	298 ± 41
$[Na^+]$ (mM)	175 ± 8	132 ± 16	106 ± 23
$[K^+]$ (mM)	4.2 ± 0.4	16 ± 7	59 ± 16
$[Cl^-]$ (mM)	132 ± 11	88 ± 9	177 ± 22

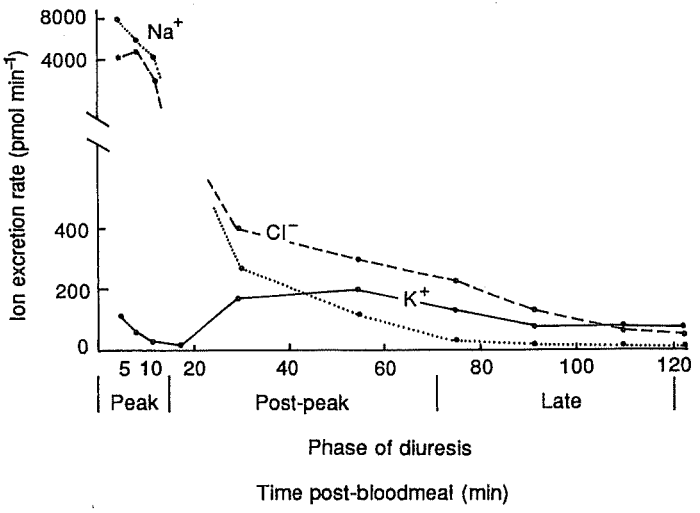

Figure 16.3 Ion excretion rates as a function of time after the start of a blood meal taken by a female *Aedes aegypti* on a human host. Assayed in urine samples collected from the individual used in Figure 16.2. The first Cl^- datum point is probably too low due to an artefact in the assay (K.W. Beyenbach, personal communication.) (After Williams *et al.*, 1983.)

bloodfed females. The osmolality of human plasma was significantly lower than that of the haemolymph, its sodium concentration was significantly higher. Williams *et al.* (1983) measured the rates of water and ion excretion of bloodfed females of *Ae aegypti*, and their observations provide an insight into the mosquito's homeostatic capabilities. Energy dispersive spectra showed Na, K and Cl to be the only elements in urine with atomic weights >20. The osmolality of the urine could be accounted for largely by the salts of sodium and potassium. These were mostly chlorides but in many urine samples the chloride concentration was less than the sum of the sodium and potassium concentrations; in one experiment the mean anion concentration deficit amounted to 47 mM during the peak phase and 60 mM during the early post-peak phase (Table 16.1B).

The three phases of diuresis were characterized not only by flow rate but also by the ionic composition and osmolality of the urine. The peak phase was characterized by rapid excretion

of a urine high in sodium, low in potassium, and with an osmolality slightly below that of haemolymph (Figure 16.3, Table 16.1). Williams *et al.* (1983) postulated that the high rate of urine flow probably prevented the hindgut from modifying the composition of its contents to any great extent, so that the urine would not differ much from the tubular fluid. Potassium excretion increased during the post-peak phase, when its concentration in urine exceeded that in human plasma (Figure 16.3, Table 16.1). This suggests that the erythrocyte cell membranes become leaky within 30 min of ingestion.

The late phase of diuresis was characterized by low rates of urine flow, a relatively high potassium excretion rate, and urine of variable osmolality. The very low urine osmolalities (<100 mosmol kg^{-1}) that were sometimes observed, and the low flow rate, suggest that fluid remained in the hindgut for relatively long periods of time. By the time that experiments were terminated, 120 min post-bloodmeal, the urine had usually become isosmotic with the haemolymph again.

Urine sodium concentration and flow rate were positively correlated; urine potassium concentration and flow rate were negatively correlated but with a strongly non-linear relationship. Potassium concentrations were low when flow rates exceeded 20 nl min^{-1} and were variable at lower flow rates, sometimes exceeding 100 mM. The urine chloride concentration showed no significant correlation with flow rate. Urine flow rate and osmolality were positively correlated. During the peak phase urine osmolality was marginally higher than that of human plasma, and slightly below the mean haemolymph osmolality but within its range (300–400 mosmol kg^{-1}). As flow rates declined the urine became increasingly hypo-osmotic to the haemolymph, falling below 100 mosmol kg^{-1} in some cases. Excretion of 'free water' occurred during the post-peak phase, when the urine was hypo-osmotic to the ingested plasma as well as to the haemolymph. When the flow rates became very low the urine became isosmotic with the haemolymph again.

In Table 16.2 the volume and electrolyte content of the blood meal are broken down into the loads contained in plasma and erythrocytes, which are dealt with by the mosquito at different times after ingestion of the blood meal, and compared with the volume and electrolyte loads excreted in urine. The excreted volume represented only 23% of the total ingested load but 42% of the ingested plasma load. Some 80% of volume reduction occurred during the first 20 min after feeding. The amount of potassium excreted exceeded the plasma potassium load. The experimental mosquitoes were estimated to contain 0.6 µl haemolymph. More than 0.6 µl of urine was excreted during the first 20 min after feeding, therefore a volume at least as great must have been absorbed from the blood meal during this period. The ultrastructure of the midgut epithelium is consonant with a transport function. During the first hour after blood feeding changes occur in the

Table 16.2 Mean volumes and electrolyte loads in the meal of human blood taken by females of *Aedes aegypti* and in the urine collected during 102 ± 10 min from the beginning of the meal. (From Williams *et al.*, 1983.)

Parameter	*Ingested load*			*Excreted load*	
	Total	*Erythro-cytes*	*Plasma*	*Urine*	*% of ingested plasma load*
Volume (µl)	3.5	1.6	1.9	0.8	42
$[Na^+]$ (nmol)	284	14	270	120	44
$[K^+]$ (nmol)	149	140	9	13	144
$[Cl^-]$ (nmol)	300	110	190	111	58

ultrastructure of the rectal papillae, notably a swelling of the terminal regions of the basal labyrinth. Ultrastructural changes continue up to 12 h post-bloodmeal and the altered state persists for another 24 h at least (Hopkins, 1967).

The capacity for fluid secretion *in vitro* by tubules of *Ae taeniorhynchus*, in response to 5-HT and cAMP, was lower in tubules taken from adults than in those from larvae. Tubules dissected from 3-day-old females immediately after blood feeding showed a significantly greater transport capacity than tubules from sugarfed females of the same age. This effect of blood feeding persisted in 4-day-old females. Unlike the situation in some other insects, the increased capacity for fluid secretion was not associated with ultrastructural reorganization of the microvillate cell membrane (Bradley and Snyder, 1989).

Anopheles freeborni showed a striking capacity for post-bloodmeal diuresis. Diuresis started immediately after cessation of feeding and continued at a constant rate, between 35 and 45 nl min^{-1} depending on the female, for almost 30 min. Diuresis ended by 45–50 min after the cessation of feeding. Females that took small blood meals discharged urine over the same time period but at a lower rate, suggesting that the rate of diuresis reflects the rate at which water and ions pass into the haemolymph from the gut. Fluid transfer from midgut to haemolymph continued for some time after diuresis had ended; the midgut continued to shrink while the volume of haemolymph around it increased proportionately (Nijhout and Carrrow, 1978).

16.2 MECHANISMS OF TUBULAR FLUID PRODUCTION

The excretory system of mosquitoes must meet complex requirements. The blood-feeding habit necessitates the rapid excretion of water and sodium ions without loss of potassium immediately after feeding. During the course of digestion, potassium ions must be eliminated at a regular slow rate. In the absence of fluid intake, salts and water must be circulated internally without loss.

16.2.1 Methods

Two types of experimental preparation have been used to investigate the properties of adult female *Aedes aegypti* Malpighian tubules – non-perfused and perfused. In the non-perfused Ramsay preparation, a tubule was severed from the alimentary canal and placed in a 50 µl drop of saline which was under oil. The open end of the tubule was pulled into the surrounding oil so that secreted fluid that flowed from the open end accumulated in the oil as an aqueous droplet (Figure 16.4A). The dimensions of the secreted droplets were measured optically; their contents could be analysed by electron-probe microdroplet techniques and wavelength dispersive spectroscopy. Test substances could be added to the bathing saline and their effects on fluid and ion secretion rates measured (Williams and Beyenbach, 1983).

The electrical properties of the Ramsay preparation could be investigated by use of a microelectrode inserted into a principal cell or inserted through cells into the tubule lumen, and with a reference electrode in the drop of bathing saline. Penetration to the tubule lumen permitted measurement of the transepithelial electrical potential difference or voltage (V_t). Impaling a primary cell permitted measurement of the basolateral membrane potential (V_{bl}). The apical membrane potential (V_a) could be estimated from the difference between V_t and V_{bl}. Membrane conductances could be evaluated from the changes of V_{bl} following changes in the bathing saline (Sawyer and Beyenbach, 1985: Aneshansley *et al.*, 1988).

In a modification of the non-perfused preparation, isolated tubules were fixed to the wax bottom of a saline flow-bath by means of their tracheal attachments. The primary cells were impaled with a microelectrode to measure membrane potentials and the microelectrode was advanced into the tubule lumen to measure V_t (Sawyer and Beyenbach, 1985).

Perfused preparations were set up by first cutting a segment 0.5–2 mm long from a Malpighian

tubule, drawing its ends into the mouths of glass holding pipettes, and submerging the whole in a saline bath of 0.3 ml capacity (Figure 16.4B). One end of the tubule segment was cannulated with a perfusion pipette (tip diameter 10–15 μm) which contained a silver wire electrode. The other holding pipette, into which perfused fluid emerged, served as a collection pipette. Alternatively the tubule segment could be cannulated with a double-barrel pipette; the tubule lumen would be perfused with saline from one barrel which also served to measure voltage, and current could be injected into the tubule lumen through the other barrel. The salines that bathed and perfused the tubule were usually identical, but test substances could be introduced into the bath saline or perfusate.

The transepithelial voltage (V_t) of perfused tubule preparations was measured directly between electrodes in contact with the tubule contents and the bath saline. The transepithelial electrical resistance (R_t) was measured by cable analysis after the injection of short pulses of current. Basolateral membrane voltage could be measured with conventional microelectrodes inserted into the principal cells. The fractional resistance of the basolateral membrane of tubule primary cells ($f_{R_{bl}}$), defined as the ratio of the basolateral membrane resistance over the sum of the basolateral (R_{bl}) and apical membrane resistances (R_a), was determined from the voltage deflection across the basolateral membrane, measured with an intracellular microelectrode after a transepithelial current pulse. Thus,

$$f_{R_{bl}} = \frac{\Delta V_{bl}}{\Delta V_t} = \frac{\Delta V_{bl}}{\Delta V_{bl} + \Delta V_a} = \frac{R_{bl}}{R_{bl} + R_a} \quad (16.1)$$

The electrical characteristics of perfused tubule preparations could be measured when the tubules were perfused with bathing saline, i.e. in the absence of transepithelial ion gradients when all transepithelial voltages arose from the transport activities of the epithelial cells (Williams and Beyenbach, 1984: Petzel *et al.*, 1985: Aneshansley *et al.*, 1988; Hegarty *et al.*, 1991).

16.2.2 Characteristics of spontaneously secreting tubules

Malpighian tubules of *Aedes aegypti* secreted fluid spontaneously as soon as they had been transferred from the mosquito into a saline droplet

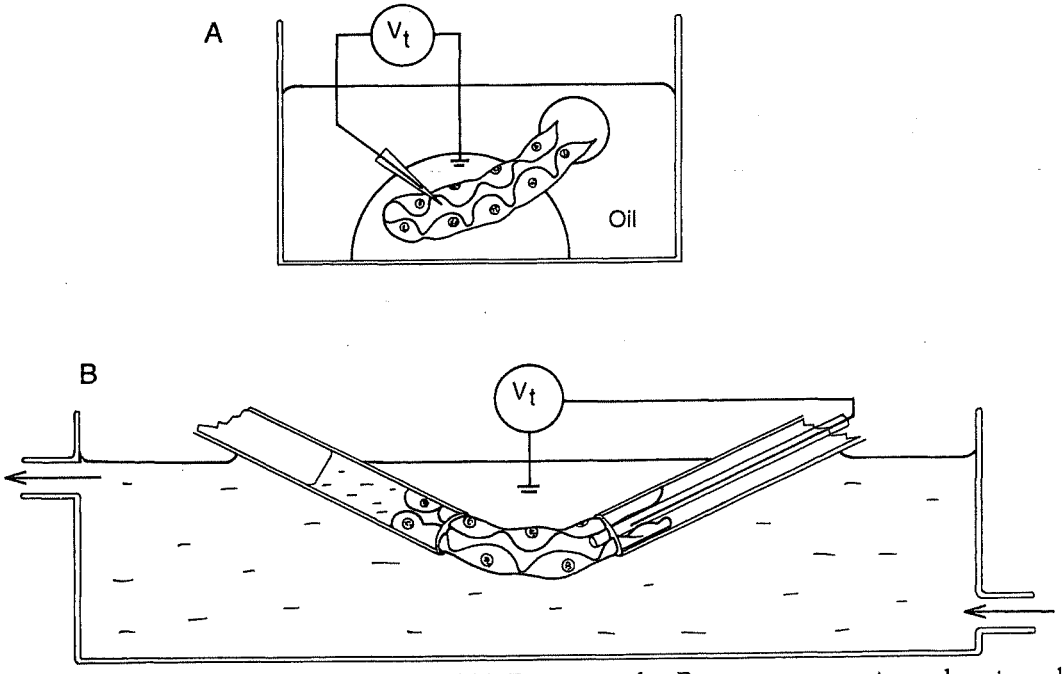

Figure 16.4 Two Malpighian tubule preparations. (A) Diagram of a Ramsay preparation, showing also penetration of the tubule with a microelectrode for measurement of the potential difference between the tubule lumen and the bathing saline. (B) Diagram of a perfused preparation. (After Aneshansley *et al.*, 1988.)

under oil. The rate of fluid secretion varied between tubules, but for any tubule the rate of secretion was constant for 5 h or more. This variation apart, no differences in electrical or secretory properties have been found between the five Malpighian tubules of individual *Ae aegypti*, consistent with their anatomical similarity. Under control conditions with Ramsay preparations, the mean rate of spontaneous fluid secretion by single tubules from adult females varied from 0.65 to 0.79 nl min^{-1}. The secreted fluid was nearly isosmotic with the bathing saline and consisted of rather similar concentrations of NaCl and KCl. Its $[Na^+]$ represented 50–60% of that of the bathing saline. The $[K^+]$ was some 30–40 times greater than, and the $[Cl^-]$ about the same as, that of the saline (Table 16.3). Na^+, K^+ and Cl^- accounted for over 90% of the measured osmolality (Williams and Beyenbach, 1983; Petzel *et al.*, 1985; Hegarty *et al.*, 1991). Such an isosmotic high-potassium fluid is secreted by most isolated insect Malpighian tubules.

Measurements of transepithelial and transmembrane voltages and resistances have been made using perfused *Ae aegypti* tubule preparations exposed to symmetrical saline solutions. Tubules consistently generated lumen-positive transepithelial voltages, ranging from *c.* 25 to 90 mV. Values of the apical membrane voltage were consistently higher than those of the basolateral membrane. Transepithelial resistance ranged between *c.* 4 and 24 kΩ.cm. Under control conditions, the values of the fractional resistance of the basolateral membrane ($f_{R_{bl}}$) and the apical membrane ($1\text{-}f_{R_{bl}}$) were usually similar (Table 16.4) (Hegarty *et al.*, 1991). In one series of experiments a mean transepithelial voltage (V_t) of 30 mV (lumen positive) was recorded. The mean basolateral membrane voltage was −77 mV and the mean apical membrane voltage −107 mV (both cell negative). The mean transepithelial resistance (R_t) was 14.9 kΩ.cm (Petzel *et al.*, 1987). Measurement of V_t in non-perfused preparations, in which the tubule lumens contained secretory fluid, revealed a positive voltage similar to that in symmetrically-perfused tubules (Sawyer and Beyenbach, 1985). The transepithelial voltage was rapidly inhibited by dinitrophenol, consistent with its being due to active transport. Williams and Beyenbach (1984) concluded, from the value of R_t and the ability of the tubules to generate transepithelial concentration gradients (Table 16.3), that the *Ae aegypti* Malpighian

Table 16.3 Electrochemical characteristics of Malpighian tubule preparations in the absence and presence of 1 mM dibutyryl cAMP, derived from fluid composition data from Ramsay preparations and electrical measurements on perfused preparations. Transepithelial potential difference (V_t). Transepithelial chemical potential difference ($\Delta\mu$). Transepithelial electrochemical potential difference ($\Delta\tilde{\mu}$) calculated as the sum of V_t and $\Delta\mu$. (From Williams and Beyenbach, 1984.)

Treatment	*Ion*	*Concentration (mM)*		V_t	$\Delta\mu$	$\Delta\tilde{\mu}$
		Bath	*Lumen*	*(mV)*	*(mV)*	*(mV)*
Control	Na^+	159	94	+53*	−13†	+ 40*
	K^+	3	91		+87*	+140*
	Cl^-	157	161		− 1*	+ 52†
cAMP	Na^+	159	178	+99*	+ 3*	+102*
	K^+	3	17		+44*	+143*
	Cl^-	157	185		− 4*	+ 95†

* Potential difference opposes secretion of the ion.
† Potential difference favours secretion of the ion.

Table 16.4 Electrophysiological characteristics of perfused *Aedes aegypti* Malpighian tubule preparations bathed and perfused with identical salines, in the absence of secretagogues (controls) and when bathed in 10^{-4} M dibutyryl cAMP in the absence or presence of 10^{-4} M bumetanide. (From Hegarty *et al.*, 1991.)

Parameter	*Control*	*cAMP*	*p*	*Control*	*cAMP + bumetanide*	*p*
Basolateral membrane						
Fractional resistance	0.56	0.32	<0.001	0.53	0.42	<0.05
Voltage (mV)*	–62.9	–33.9	<0.001	–55.2	–23.0	<0.001
Apical membrane						
Voltage (mV)*	110.5	105.6	n.s.	92.7	90.3	n.s.
Transepithelial						
Resistance (kΩ.cm)	14.3	9.8	<0.01	9.8	7.9	<0.01
Voltage (mV)†	47.6	71.8	<0.05	37.5	67.2	<0.001

*Cell negative.
†Lumen positive.
n.s., not significant

tubule should be classified as a moderately tight epithelium.

The passive permeabilities of tubules were investigated by measurements of transepithelial diffusion potentials, observed following changes of ion concentration. Lowering the bath sodium concentration from 159 to 9 mM caused a drop of 23 ± 6 mV in V_t, whereas lowering the luminal sodium concentration had no significant effect. Equally substantial changes to bath or lumen chloride concentrations had no effect on transepithelial voltage. Williams and Beyenbach (1984) concluded that bath [Na^+] was important for the maintenance of V_t.

Conductances of the basolateral membrane were investigated by measuring the effects on basolateral membrane voltage (V_{bl}) of changes in the ion concentrations of the bathing saline. A 5-fold decrease in bath [Na^+] hyperpolarized the membrane (cell negative) by 10 mV whereas a 4.4-fold increase in bath [K^+] depolarized it by 8 mV. The study revealed that the basolateral membrane was permeable to Na^+ and K^+ with both conductances of similar magnitude, consistent with the similar Na^+ and K^+ secretion rates measured under control conditions. The intracellular electrical potential appeared to be dominated by transmembrane diffusion potentials for K^+, judging by the negative V_{bl} and the depolarization induced by raised bath [K^+] (Sawyer and Beyenbach, 1985).

Only little is known about the apical membrane. Experimental evidence consistent with the presence of an electrogenic pump, presumably a H^+-ATPase, in the apical membrane of the principal cells of *Ae aegypti* tubules was reported by Beyenbach and Pannabecker (1991) and Pannabecker *et al.* (1992). Evidence consistent with the presence of chloride channels in the apical membrane was obtained with patch-clamp methods. The putative chloride channels had 25 pS conductance in symmetrical salines (160 mM-Cl^-). With asymmetrical salines the reversal potentials of the single channel current suggested that the channels were highly selective for Cl^- (Wright and Beyenbach, 1987).

The fractional resistance of the basolateral membrane ($f_{R_{bl}}$), i.e. the ratio of the basolateral membrane resistance to the total transcellular resistance, was 0.63 ± 0.11. This value of 63% indicated that under control conditions the conductance of the apical membrane was greater than that of the basolateral membrane. Because the fractional resistance was a function of membrane specific resistance and membrane surface area this was not a surprising result given the microvillate nature of the apical membrane (Petzel *et al.*, 1987).

Williams and Beyenbach (1984) calculated the transepithelial electrochemical potential differences ($\Delta\tilde{\mu}$) for Na^+, K^+ and Cl^- as the sum of the transepithelial voltage (V_t) and the transepithelial chemical potential difference ($\Delta\mu$) (Table 16.3). From the values of $\Delta\tilde{\mu}$ they concluded that in the absence of secretagogues both sodium and potassium were secreted against their respective electrochemical gradients, whereas chloride movement could be explained by passive secretion. It was not possible to estimate the rate of chloride secretion that could be driven by V_t because the magnitude of the chloride conductance was not known.

Generally, epithelial tissues that function in isosmotic water and ion transport have the ultrastructure and electrochemical properties characteristic of 'leaky epithelia', i.e. a microvillate apical cell membrane and infolded basal cell membrane, low transepithelial voltage and resistance, small transepithelial ionic and osmotic gradients, and high permeability to solutes and water. The Malpighian tubules of *Aedes aegypti* are remarkable in combining the ultrastructural characteristics and capacity for high rates of salt and water transport of a 'leaky epithelium' with the electrochemical characteristics of a moderately 'tight epithelium', i.e. high transepithelial voltage, moderate transepithelial electrical resistance, and significant transepithelial sodium and potassium gradients. On balance the mosquito Malpighian tubule is foremost a transporting epithelium; it secretes Na^+ and K^+ by active transport across an epithelium which is nearly impermeable to the electrodiffusion of Na^+, K^+ and Cl^- but highly permeable to water (Maddrell, 1980; Aneshansley *et al.*, 1988).

Studies with *Aedes taeniorhynchus* showed that the secretory characteristics of the adult Malpighian tubules differ from those of the larvae. In Ramsay preparations the sodium concentration of the tubular fluid was positively correlated with that of the bathing medium (Figure 16.5). However, the tubular fluid secreted by adult tubules was markedly richer in sodium than that secreted by larval tubules, although the larvae have a striking capacity for sodium excretion, being adapted to the saline waters of salt marshes. Adult female tubules secreted five times faster than the smaller male tubules, and female tubular fluid was significantly richer in sodium than that of males. Larvae of *Ae taeniorhynchus* reared in sulphate-enriched water had a striking ability to secrete sulphate at very high concentration (Section 6.5.3(a)), but this ability was absent from male and female adults derived from such larvae (Maddrell, 1977; Maddrell and Phillips, 1978).

The rate of fluid secretion by tubules of adult *Ae taeniorhynchus* was sensitive to potassium concentration. When the bathing saline $[K^+]$ was 5–10 mM (said to be the concentration in haemolymph) adult female tubules secreted 1–2 nl fluid per minute, but in 44 mM-K^+ the secretion rate was 5 nl min^{-1}. Maddrell (1977) thought this inappropriate since, under the conditions obtaining in haemolymph, fluid secretion would be considerably below the maximum rate; however, the measurements were made in the absence of excretagogues.

16.2.3 Responses to cyclic AMP

The functional importance of cAMP in *Aedes aegypti* Malpighian tubules was shown by the striking effects of dibutyryl cyclic AMP (db-cAMP), a

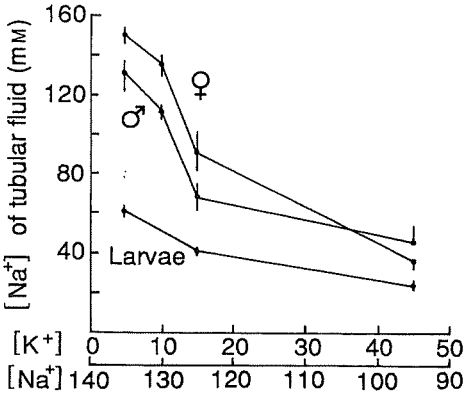

Figure 16.5 The sodium concentration of tubular fluid secreted by Malpighian tubules from larvae and male and female adults of *Aedes taeniorhynchus* when subjected to bathing salines of different sodium and potassium concentrations. Means ± s.e. (From Maddrell, 1977.)

membrane-permeable derivative. Addition of 0.1 or 1 mM db-cAMP to the bath rapidly changed the resistance and voltage profiles of the tubular epithelium (Table 16.4). The principal effect was a reduction in the fractional resistance of the basolateral membrane and a consequent depolarization of the basolateral membrane voltage. Because the apical membrane fractional resistance and voltage were unaffected, the transepithelial resistance decreased and the transepithelial voltage hyperpolarized ($V_t = V_{bl} + V_a$). The cAMP-induced hyperpolarization of V_t was reduced by a lowering of bath [Na^+], but was not affected by a lowering of bath [Cl^-], showing that cAMP increased the basolateral sodium conductance (Williams and Beyenbach, 1984; Sawyer and Beyenbach, 1985; Petzel *et al.*, 1987; Hegarty *et al.*, 1991).

When 0.1 or 1 mM db-cAMP was included in the saline that bathed Ramsay preparations, the rate of fluid secretion promptly increased. Electron probe analysis of the secreted fluid revealed that the exogenous cAMP had significantly increased the luminal [Na^+] and significantly decreased the luminal [K^+] without significantly affecting the luminal [Cl^-] (Table 16.5A). Because the fluid secretion rate increased in the presence of exogenous cAMP, the effects of cAMP on net ion secretion rates, calculated as the product of fluid secretion rates and ion concentrations, differed from its effects on luminal ion concentrations: sodium and chloride secretion rates were significantly enhanced but the rate of potassium secretion was little changed (Table 16.5B) (Williams and Beyenbach, 1983; Hegarty *et al.*, 1991). The primary cells were estimated to secrete the equivalent of their sodium content more than ten times per minute when stimulated with cAMP (Aneshansley *et al.*, 1988).

In the presence of db-cAMP, as under control

Table 16.5 Ion concentrations (A) and fluid and ion secretion rates (B) of tubular fluid secreted by isolated Malpighian tubules of female *Aedes aegypti* prepared by Ramsay's method. (From Williams and Beyenbach, 1983; and Petzel *et al.*, 1985.)

(A) Mean ion composition of tubular fluid from tubules bathed in saline without secretagogue (control) or containing dibutyryl cAMP (1 mM) or natriuretic factor.

Ion	*Concn. in saline (mM)*	*Concentration in tubular fluid (mM)*			
		Control	*cAMP*	*Control*	*Natriuretic factor*
Na^+	159	94	178*	76	148*
K^+	3	91	17*	114	31*
Cl^-	157	161	185	181	181

(B) Fluid and ion secretion rates of tubules bathed in saline without secretagogue (control) or in saline containing dibutyryl cAMP (1 mM) or natriuretic factor.

Fluid/Ion	*Secretion rate*			
	Control	*cAMP*	*Control*	*Natriuretic factor*
Tubular fluid (nl min^{-1})	0.79	2.9*	0.65	2.3*
Na^+ (pmol min^{-1})	69	454*	51	344*
K^+ (pmol min^{-1})	68	43	75	68
Cl^- (pmol min^{-1})	118	451*	119	416*

* Significantly different from control.

conditions, both sodium and potassium were secreted against their respective electrochemical gradients; only chloride secretion could be explained by passive movement (Table 16.3). No transepithelial chloride diffusion potentials were observed when transepithelial chloride concentration differences were imposed, confirming that there was no significant transepithelial electrodiffusion of Cl^- (Williams and Beyenbach, 1984).

That intracellular cyclic AMP ($cAMP_i$), generated within the tubule cells, functioned in the regulation of fluid secretion was demonstrated by the actions on Ramsay preparations of forskolin (a non-specific stimulant of the adenylate cyclase system) and theophylline (an inhibitor of phosphodiesterase). These secretagogues significantly increased both $[cAMP]_i$ and the rate of fluid secretion. In the absence of secretagogues $[cAMP]_i$ was 340 pmol mg protein^{-1} and the fluid secretion rate 0.56 nl min^{-1}. Fluid secretion rates reached their mean maximum of about 2 nl min^{-1} when $[cAMP]_i$ was driven to 900 pmol mg protein^{-1}; driving $[cAMP]_i$ higher with additional secretagogue did not stimulate the secretion rate further (Petzel *et al.*, 1987).

Bumetanide is an inhibitor of electrically neutral Na^+–K^+–$2Cl^-$ co-transport. When applied alone to *Ae aegypti* tubules, 0.1 mM bumetanide significantly increased the $[Na^+]$ and decreased the $[K^+]$ in secreted fluid, but because the increase in $[Na^+]$ was of similar magnitude to the decrease in $[K^+]$ there was no net change in cation concentration, and hence none in anion concentration or in rate of fluid secretion. However, when applied with db-cAMP bumetanide was a potent inhibitor of cAMP-stimulated transepithelial electrolyte and fluid secretion.

Exposure of tubules to db-cAMP plus bumetanide caused greater depolarization of the basolateral membrane than exposure to cAMP alone, but it produced no increased effect on the basolateral membrane fractional resistance, and the transepithelial resistance fell by 19% rather than 31% (Table 16.4). These results suggest that the actions of bumetanide are electrically neutral, and indicate that the electrophysiological effects of db-cAMP, like the secretagogue effects, depend on the presence of an intact bumetanide-sensitive transport system.

16.2.4 Peptides, eicosanoids and pharmacological agents

The addition of a crude saline extract of mosquito heads to the bathing saline of an isolated Malpighian tubule altered its electrophysiological characteristics, stimulated fluid secretion, and significantly increased the rates of sodium and potassium secretion (Williams and Beyenbach, 1983, 1984). Interpretation of these results was impossible because of the wide range of pharmacologically active substances in the extract, consequently attempts were made to isolate and purify peptide neurohormones that were believed to be present. HPLC of saline extracts from the heads of 3–10-day-old male and female *Aedes aegypti* yielded three fractions that affected the transepithelial voltage of perfused tubules. Their active constituents were shown to be peptides with molecular weights between 1.8 and 2.7 kDa (Petzel *et al.*, 1985).

The actions of fractions I, II and III on tubule preparations, which were rapidly reversible on wash-out from the bath, are summarized in Table 16.6. Fraction I depolarized V_t but had no effect on $[cAMP]_i$ nor upon the rates of fluid or ion secretion *in vitro*. Fraction II depolarized V_t, caused a slight but significant increase in $[cAMP]_i$, stimulated the rate of fluid secretion 3- to 4-fold, and increased the sodium and chloride secretion rates. It did not alter the potassium secretion rate but it significantly reduced $[K^+]$ in the tubular fluid. Fraction III hyperpolarized V_t after an initial transient depolarization, caused a massive increase in $[cAMP]_i$, and had the same effects on fluid and ion secretion as fraction II. As far as they were examined, the actions of fraction III on the tubule primary cells were almost identical with those of dibutyryl cAMP, and it was suggested that cAMP had the role of second messenger to fraction III. Because fraction III stimulated secretion of NaCl-rich

as opposed to KCl-rich fluid (Table 16.5), the name **mosquito natriuretic factor** was proposed for it (Petzel *et al.*, 1985, 1986, 1987).

To examine their actions *in vivo*, fractions I, II and III were injected into females that had been bloodfed and immediately decapitated to prevent (further) release of endogenous diuretic hormone. All three fractions significantly increased the volume of urine discharged compared with the saline-injected controls. Fraction I showed the lowest activity, fraction III the greatest (Wheelock *et al.*, 1988).

These results suggest that the active peptide in fraction III is a natriuretic hormone which activates the adenylate cyclase system in Malpighian tubules and thereby raises the internal cyclic AMP concentration, increases the sodium conductance of the basolateral membranes, and so stimulates secretion of an NaCl-rich fluid (Petzel *et al.*, 1987).

Aedes aegypti Malpighian tubules are responsive to leucokinins, a family of octapeptides present in the head of the cockroach *Leucophaea maderae* and which increase the motility of cockroach hindgut. Exposure of perfused *Ae aegypti* tubules to seven of the leucokinins at 3.6×10^{-7} M caused a fall in transepithelial voltage in each case; leucokinin-4 was active at 3.6×10^{-9} M. Depolarization was dependent on extracellular chloride concentration. In tests with five of the peptides, lowering bath [Cl^-] to 10 mM greatly reduced the leucokinin-induced depolarization. At concentrations between 10^{-11} and 10^{-9} M, leucokinin-8 significantly reduced the rate of fluid secretion but had no effect on transepithelial voltage. At concentrations of 10^{-8} M or more it depolarized the tubules, and at 10^{-7} M or more it increased the rate of fluid secretion. Hayes *et al.* (1989) pointed to similarities between the actions of fraction II and the leucokinins on mosquito tubules (Table 16.6) and postulated that fraction II and the leucokinins might be structurally related.

Subsequently, peptides that depolarized the Malpighian tubule transepithelial voltage were isolated from mosquitoes. They bore structural similarities to the leucokinins (Section 10.3.1 (b)), and were designated culekinin depolarizing peptides (CDP). The ED_{50} of CDP-I for tubule depolarization was 2×10^{-10} M, and this action was chloride dependent (Hayes *et al.*, 1992; T.K. Hayes, personal communication).

A peptide in *Ae aegypti* head extract exhibited antidiuretic activity. It decreased fluid secretion by tubules in Ramsay preparations by 76%, reduced forskolin-stimulated fluid secretion by 67%, and caused a 21 mV hyperpolarization of transepithelial voltage (Petzel and Conlon, 1991).

Eicosanoids (oxygenated metabolites of certain C_{20} polyunsaturated fatty acids) regulate many aspects of mammalian renal function. Exposure of *Ae aegypti* Malpighian tubule preparations to 0.1 mM concentrations of specific inhibitors of eicosanoid biosynthesis reduced basal fluid secretion rates. ETYA (5,8,11,14-eicosatetraynoic acid), which

Table 16.6 Actions upon perfused Malpighian tubule preparations of three HPLC-fractions of *Aedes aegypti* head extract, dibutyryl cAMP and two peptides. (From the data of Williams and Beyenbach, 1983, 1984; Petzel *et al.*, 1985, 1987; Wheelock *et al.*, 1988; Hayes *et al.*, 1989, 1992.)

Test substance	*Transepithelial voltage*	*Is effect on voltage Cl^- dependent*	*$[cAMP]_i$*	*Rate of transepithelial fluid secretion*
Fraction I	Depolarized	nt	Unchanged	Unchanged
Fraction II	Depolarized	nt	Raised	Stimulated
Fraction III*	Hyperpolarized	nt	Raised	Stimulated
cAMP (1 mM)	Hyperpolarized	No		Stimulated
Leucokinin-8	Depolarized	Yes	nt	Inhibited/Stimulated†
CDP-I	Depolarized	Yes	nt	Stimulated

nt, not tested
* Mosquito natriuretic factor
† Dose Dependent action

inhibits all eicosanoid biosynthesis, reduced fluid secretion by more than 50%. Esculetin, a lipoxygenase inhibitor, had little or no effect on fluid secretion, but the epoxygenase inhibitor SKF525A produced a small but significant reduction in fluid secretion. Two competitive inhibitors of cyclooxygenase, indomethacin and naproxin, yielded similar results to ETYA. The effects of all eicosanoid biosynthesis inhibitors were reversed after washing and upon addition of cAMP. Petzel and Stanley-Samuelson (1992) concluded that products of the cyclooxygenase pathway, in particular prostaglandins, modulate basal rates of fluid secretion by Malpighian tubules.

Addition of a pulse of 250 pmol 5-hydroxytryptamine to the irrigating saline of a perfused tubule preparation caused a transient depolarization of the transepithelial potential. With larger doses the depolarization was followed by a brief hyperpolarization. Irrigation of a Ramsay preparation with 10^{-6} M 5-HT depolarized the transepithelial voltage almost to zero and weakly stimulated fluid secretion. Irrigation with 10^{-5} M 5-HT caused a slight but significant increase in $[Na^+]$ in the tubular fluid and a similar decrease in $[K^+]$, while $[Cl^-]$ remained unchanged (Veenstra, 1988).

Exposure of Ramsay preparations to 10^{-4} M ouabain for 30 min significantly inhibited the rate of fluid secretion, indicating the presence on the basolateral membrane of an Na^+–K^+ pump (Hegarty *et al.*, 1991).

16.2.5 Characteristics of male tubules

In Ramsay preparations, Malpighian tubules from male *Aedes aegypti* had a basal secretory rate one-sixth that of female tubules. The concentrations of Na^+, K^+ and Cl^- in the tubular fluid were broadly similar in the two sexes. Extracts of male and female heads generally had the same effects upon Malpighian tubules in terms of sodium, chloride and water secretion, and of transepithelial voltage and resistance, whether the tubules were male or female. Differences were observed between male and female tubules in the secretion of potassium in response to partly purified head extracts but might have been artefactual. Cyclic AMP stimulated sodium, chloride and water secretion by male tubules as it did with female tubules. Plawner *et al.* (1991) concluded that the Malpighian tubules of males and females had similar ion-transport mechanisms for NaCl and fluid secretion and that the diuretic hormones and receptors were also similar. Quantitative differences in secretion rates reflected the larger size of the female tubules.

16.2.6 Models of tubular fluid secretion

To satisfy the separate requirements of Na^+ discharge during post-bloodmeal diuresis and K^+ production from erythrocytes during digestion, any model of tubular fluid secretion must permit independent processing of Na^+ and K^+. For our purpose, we shall adopt the theoretically valid but unproven concept that the driving force in fluid secretion is an active transfer of cations across the apical (luminal) face of the cell, and that the presence in the tubule lumen of high concentrations of Na^+, K^+ and Cl^- draws water out of the cell by osmosis (Maddrell, 1991). Cation transport generates lumen-positive voltages, and anion transport generates negative voltages, hence the transepithelial voltage reflects the relative rates of cation and anion transport. If the dominant driving force for NaCl and KCl transport is active transport of cations, the transepithelial voltage will be lumen-positive, and increasing the rate of cation transport will induce hyperpolarization of the transepithelial voltage. Thermodynamic data indicate that isolated, spontaneously-secreting mosquito Malpighian tubules secrete Na^+ and K^+ from the bath to the tubule lumen by active transport and Cl^- by passive transport. However, different peptides extracted from mosquitoes can hyperpolarize or depolarize the tubule transepithelial voltage, therefore it is likely that more than one model of tubular fluid secretion is needed.

A tentative model of ion movements in the Malpighian tubules of *Aedes aegypti*, under conditions of spontaneous secretion and of stimulation by natriuretic factor, is illustrated in Figure 16.6. Experimental evidence has provided preliminary

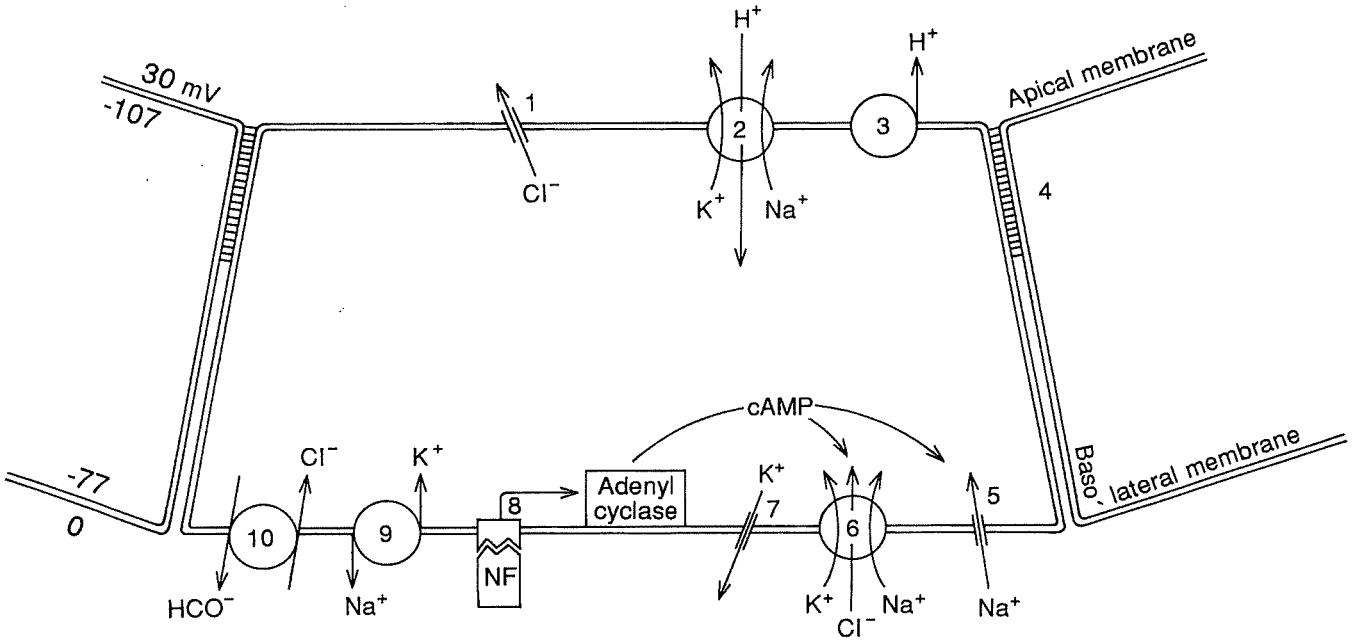

Figure 16.6 Tentative model of mechanisms controlling movement of ions through the principal cells of the Malpighian tubules of adult female *Aedes aegypti*. Representative voltages are indicated. 1. Postulated chloride channel. 2. Postulated antiport ion exchanger. 3. Postulated H^+-ATPase. 4. Smooth septate junction. 5. Sodium channel. 6. Postulated bumetanide-sensitive Na^+–K^+–$2Cl^-$ carrier. 7. Potassium channel. 8. Receptor for natriuretic factor. 9. Na^+/K^+ pump, ouabain sensitive. 10. Postulated SITS-sensitive Cl^-/HCO_3^- exchange.

evidence for a variety of transport systems in the basolateral membrane. The existence of sodium and potassium channels was shown by the dominant K^+ conductance and by the smaller Na^+ conductance which increased in the presence of cAMP. The inhibition of fluid secretion by ouabain revealed the presence of a Na^+/K^+ carrier. The fact that db-cAMP lowered the fractional resistance of the basolateral membrane and increased the basolateral sodium conductance suggests that cAMP selectively activated certain ion channels. The antagonistic effects of bumetanide indicate that the actions of cAMP were in part mediated by bumetanide-sensitive transport, possibly a Na^+–K^+–$2Cl^-$ co-transport system. There is circumstantial evidence for the presence of SITS-sensitive sites of chloride entry, which possibly act via Cl^-/HCO_3^- exchange.

Stimulation of the principal cells by natriuretic factor caused cAMP synthesis and a consequent increase in sodium, chloride and fluid secretion rates. A number of lines of evidence suggest that under stimulated conditions the entry of sodium, potassium and chloride ions is linked – for example, the relationship of sodium and potassium secretion rates to bath chloride concentration, and the action of bumetanide on cAMP stimulated cells. It is thought that cAMP enhances both the rate of Na^+ entry via sodium channels and the entry of Na^+, K^+ and Cl^- via the electrically neutral Na^+–K^+–$2Cl^-$ carrier. Efflux of K^+ through potassium channels in the basolateral membrane possibly limits potassium secretion by stimulated tubules.

Because cAMP increased the rate at which sodium was secreted against its transepithelial electrochemical gradient, and caused an increase in V_t that was dependent upon bath $[Na^+]$, it appears that cAMP-stimulated sodium secretion, coupled with the passive movement of chloride, is the prime factor responsible for increases in the rate of fluid secretion in post-bloodmeal diuresis. We may conclude that the natriuretic factor present in fraction III is a genuine diuretic agent, selectively stimulating the secretion of Na^+, Cl^- and water.

The leucokinins and culekinins are much weaker diuretic agents and their effects are non-specific, increasing the secretion of Na^+, K^+ and

Cl^-. These peptides depolarize the transepithelial voltage, apparently by increasing transepithelial Cl^- conductance. Any increase in permeability of the tubule wall to Cl^- would increase the availability of Cl^- for secretion with Na^+ and K^+ and would increase the rate of fluid secretion. The mechanisms by which leucokinins and culekinins modulate Cl^- conductance, and the site of the Cl^- conductance in the epithelium, remain to be elucidated.

The inhibition of potassium secretion by bumetanide suggests that the Na^+–K^+–$2Cl^-$ carrier is involved in potassium entry into the cell. In unstimulated tubules, the Na^+–K^+–$2Cl^-$ carrier has a significant role in potassium secretion but only a minor role in sodium and chloride secretion. The ouabain-sensitive Na^+/K^+ pump is also likely to have a role in selective potassium secretion. The increased fluid secretion rate observed on treatment with ouabain is consistent with the Na^+ that entered the cell via the Na^+–K^+–$2Cl^-$ carrier not being returned to the haemolymph through the ouabain-sensitive pump. The resulting increased internal ion concentration would raise the rate of ion transport across the luminal membrane, inducing faster water flow.

For insects generally, little is known about transport systems in the apical (luminal) membrane of Malpighian tubules. The idea that sodium and potassium are actively extruded into the tubule lumen by a cation pump situated in the apical membrane has been superseded by the concept of the extrusion of hydrogen ions by a H^+-ATPase. Associated with the ATPase are antiport systems, ion-exchangers that allow H^+ to re-enter the cell in exchange for Na^+ and/or K^+. The potential difference across the luminal membrane caused by the extrusion of H^+ induces the passive outward movement of Cl^-, and the high osmotic concentration in the tubule lumen draws water out of the cell (Maddrell, 1991). For mosquito tubules, it can be stated that there is preliminary evidence of the presence of H^+-ATPase and of chloride channels in the apical membrane (Section 16.2.2). The characteristics of mosquito-tubule transepithelial electrochemical potentials indicate that Na^+ and K^+ secretion involves active processes whereas Cl^- moves passively across the apical membrane, serving electroneutrality as the counter ion of Na^+ and K^+ transport.

16.3 REGULATION OF DIURESIS

Pharmacological evidence suggests that mosquitoes, like other insects, have diuretic and antidiuretic hormones, and a number of questions naturally follow from this. Where are these hormones synthesized, and where released? How is their release regulated, and what are their actions on the target organs? Experimental investigations have taken us a short way towards the answers to these questions and suggest that, as in the larva, water movement is regulated by the combined actions of hormones and the mechanical activities of parts of the alimentary canal.

Every 3–5 s weak antiperistaltic waves passed along the posterior midgut of unfed females of *Aedes aegypti*. Every 2 or 3 s an antiperistaltic wave in the pylorus forced fluid into the posterior midgut. Then a peristaltic wave which travelled from the pylorus to the rectum drove fluid posteriorly through the hindgut lumen. The pylorus thus functioned as a two-way pump. After engorgement the antiperistaltic waves of the posterior midgut ceased and did not resume until the midgut was empty (Howard, 1962). The contractions of the hindgut of *Ae aegypti* were described by Odland and Jones (1975) as strong, myogenic and rhythmic with a frequency of 10–15 min^{-1}. Contractions started at the anterior end of the pyloric chamber and developed into peristaltic waves which swept over the anterior intestine, stopping at the junction with the rectum. Each peristaltic wave was normally succeeded by a smaller antiperistaltic wave. During diuresis, peristaltic waves passed along the rectum and anal canal. When they reached the end of the anal canal the anus opened and a drop of urine was discharged on to the postgenital lobe (Stobbart, 1977).

Abdominal distension could be the initial stimulus that leads to the induction of diuresis. When females of *An freeborni* were fed saline they

discharged urine over the same time-course as bloodfed females, but injection of 2–4 μl of saline into the haemocoele failed to induce the rapid phase of diuresis. Instead fluid was discharged at a slow rate for several hours (Nijhout and Carrow, 1978). This points to the gut rather than the body wall as the site of putative stretch receptors.

Extracts of the central nervous system have been tested for diuretic hormone. Heat-treated homogenates of heads and of thoracic ganglia of *Anopheles freeborni* proved to be equally effective in stimulating diuresis in Ramsay preparations but were inactive when injected into intact females (Nijhout and Carrow, 1978). Heat-treated head extract from *Aedes aegypi* induced diuresis in Ramsay preparations and also when injected into bloodfed decapitated females; three chromatographic fractions of head extract were active *in vitro* and *in vivo* (Table 16.6) (Wheelock *et al.*, 1988).

Bloodfed females were decapitated in investigations into the importance of the head for the control of diuresis. When females of *Ae aegypti* were decapitated soon after feeding, the rates of urine production first measured for the operated females were substantially lower than the control and pre-decapitation rates, and total urine production amounted to only 15–20% of the control quantity (Stobbart, 1977; Wheelock *et al.*, 1988). When females of *An freeborni* were decapitated 1 min after blood feeding the first measurements of urine production by the decapitated females were substantially below the control values in most cases. Urine was subsequently produced at the constant low rate of 6–7.5 nl min^{-1} for 5–7 h, by which time the operated females had discharged as much as the controls. When females were decapitated 10 or 20 min after feeding diuresis continued at the control rate for an additional 1 min and then declined to a low rate (Nijhout and Carrow, 1978). These results can be interpreted in terms of disappearance of a diuretic hormone. Clearly the head is necessary to maintain high rates of diuresis.

To find whether diuretic hormone was continuously present in the haemolymph or was released after blood feeding, haemolymph from sugarfed and bloodfed *Ae aegypti* was tested for its effect on the transepithelial voltage of perfused tubules and on the urine flow rate of Ramsay preparations. Haemolymph from bloodfed females consistently and significantly stimulated fluid secretion whereas haemolymph from sugarfed females was inactive. However, both categories of haemolymph affected the transepithelial potential (V_t). The identity of the depolarizing agent was unknown but Wheelock *et al.* (1988) concluded that a diuretic hormone was released into the haemolymph only after blood feeding, acting on the Malpighian tubules to stimulate fluid secretion.

Pinching the nerve cord of *Ae aegypti* in the 1st abdominal segment 2–4 min after blood feeding caused an immediate fall in the rate of urine flow, and pinching the anterior midgut had a similar effect. On the grounds that the site of midgut injury was posterior to the release sites of diuretic hormones, Stobbart (1977) concluded that damage to the stomatogastric nervous system must have been the cause of the failure of diuresis. He postulated that, as in the larva (Section 6.3.1), active diuresis required a change from antiperistalsis to peristalsis in the midgut musculature.

In further experiments Stobbart (1977) pinched the ventral nerve cord in the 1st abdominal segment before blood feeding, measured the diuretic rate of operated bloodfed females for a few minutes and then blocked the alimentary canal between the midgut and pyloric chamber by pressure from a blunt needle. In 12 operated but dummy-clamped females the low diuretic rate induced by pinching the nerve cord was unaffected by clamping, but in 7 out of 29 operated and clamped females the diuretic rate increased significantly, on average 4-fold, after clamping. Stobbart concluded that in unfed females tubular fluid normally moved forwards into the midgut for recycling to the haemolymph, and that in bloodfed females a change, under nervous control, from predominantly antiperistaltic to peristaltic contractions was an important element in post-bloodmeal diuresis.

Cyclic AMP almost certainly plays a role in the induction of the short post-bloodmeal burst of diuresis, which is characterized by urine with

a high sodium concentration. The intracellular cAMP concentration of the Malpighian tubules increases soon after the start of blood feeding, rising from 340 pmol to peak at 550 pmol mg protein^{-1} after about 5 min, and preceding the peak rate of NaCl diuresis by 1 min. It then declines to the control level at 9 min, before rising again to a second apparent peak at 25 min (Petzel *et al.*, 1987). Treatment of Malpighian tubule preparations with the natriuretic factor stimulated an increase in $[cAMP]_i$ and production of fluid with a high sodium concentration, and injection of this factor into bloodfed decapitated females stimulated diuresis (Table 16.6). The natriuretic factor could be the hormone responsible for the post-bloodmeal burst of diuretic activity.

Whether the phase of increased potassium excretion that occurs from 30–70 min after feeding is stimulated by a different hormone or is simply a stage in the return to the normal secretion of tubular fluid is quite unclear. It has been suggested that 'fraction I', which stimulated fluid secretion in mosquitoes decapitated after a blood meal but did not stimulate secretion in isolated Malpighian tubules, might act by inhibiting fluid resorption by the rectum (Wheelock *et al.*, 1988).

16.4 EXCRETION AND DEFAECATION AFTER EMERGENCE

Newly emerged mosquitoes contain meconium in the posterior half of the stomach in the form of fluid and semi-solid matter surrounded by peritrophic membrane. The meconium includes residues from the histolysis of the larval alimentary canal and nitrogenous wastes (Romoser, 1974) and retains chymotrypsin and trypsin activity (Briegel, 1983). The fluid discharged by *Aedes aegypti* immediately after emergence is clear but contains some uric acid. The mean weight of uric acid discharged during the first 45 min after emergence was 0.07 μg, and it did not differ significantly between males and females (DeGuire and Fraenkel, 1973; Jones and Brandt, 1981). Various species void the semi-solid meconium during the first 1–2 days after emergence, the time of discharge varying with temperature, but remnants of peritrophic membrane can persist long after the meconium has disappeared (Rosay, 1961; Venard and Guptavanij, 1966; Romoser and Cody, 1975).

During the first 48–72 h after the emergence of *Ae aegypti* uric acid was the major nitrogenous substance discharged. The excreta of sugarfed adults of *Aedes*, *Culex* and *Anopheles* contained uric acid, urea, ammonia, protein and amino acids. Older sugarfed mosquitoes excreted little nitrogenous matter; over a 5-week period the nitrogen content of sugarfed female *Ae aegypti* remained more or less constant (Terzian *et al.*, 1957; Irreverre and Terzian, 1959; Thayer and Terzian, 1971).

Most uric acid is voided via the Malpighian tubules and hindgut but some accumulates within fat body cells. Little uric acid was found in the fat body of young adults of *Culex pipiens* but the uric acid deposits increased with age and were particularly extensive in hibernating females (de Boissezon, 1930b).

16.5 COMPOSITION OF EXCRETA AND FAECES AFTER BLOOD FEEDING

Mosquitoes ingest considerably more protein in a blood meal than is subsequently found in their mature ovaries. This is because the amino acid balance of blood protein limits the amount that can be converted to yolk protein and because some blood protein must be utilized for lipid synthesis. In consequence much of the ingested protein is deaminated and the surplus nitrogen excreted in metabolites with a high nitrogen content and low molecular weight, *viz.* uric acid ($C_5H_4N_4O_3$), urea (CH_4N_2O) and ammonium ion (NH_4^+). In *Aedes* and *Culex* all surplus nitrogen from the blood meal is voided, but in *Anopheles* some appears to be retained as extra-ovarian protein (Section 22.1.1(b)).

Little is known about the transport of nitrogenous metabolites in mosquitoes, only that after blood feeding minute uric acid granules appear in the lumens of the Malpighian tubules and that the rectum progressively fills with spherical

masses and granules of uric acid (Wigglesworth, 1932).

Early investigators identified the nitrogenous metabolites present in the excreta and faeces but they were unable to account for all ingested nitrogen (Briegel, 1969; Thayer and Terzian, 1971; Thayer *et al.*, 1971; France and Judson, 1979). However, when gaseous ammonia was trapped all nitrogen in the blood meal could be accounted for from the amounts recovered in the excreta, faeces and mature ovaries (Table 16.7; Figure 16.7). Because excretion and defaecation occur at different times after blood feeding the excreta and faeces can be analysed separately.

When females of *Ae aegypti* fed rat blood were denied access to water during the period of digestion the major constituent by weight of the excreta was uric acid, with ammonium ion second and urea third. The ratios differed when computed as mol per female, when ammonium ion became the major constituent (Table 16.7). If the females were given access to water during the period of digestion their metabolism changed somewhat, with the result that more urea was produced at the expense of uric acid and ammonium ion. Uric acid, urea and ammonium ion were voided during the same time period, therefore the uricotelic, ureotelic and ammonotelic pathways operated simultaneously. The variability in the extent of ureotely revealed a degree of plasticity in excretory metabolism. The molar ratios of the various nitrogenous metabolites varied slightly with host identity (Figure 16.8). Despite its nucleated erythrocytes chicken blood gave rise to less uric acid than did rat blood (Briegel, 1986a).

After feeding on chicken blood females of *Culex quinquefasciatus* discharged 0.048, 0.096 and 0.036 μmol total amino acid on the 1st, 2nd and 3rd days post-bloodmeal, which constituted 16% of the recovered nitrogen. The ratio of free to conjugated amino acids was approximately 1:1, and most was discharged over the same period as uric acid. During the 1st and 2nd days histidine constituted 70% of the free amino acids and glycine constituted >60% of the conjugated amino acids. Arginine was present only in traces (Briegel, 1969). In *Aedes aegypti* 8–10% of discharged nitrogen was in the form of amino acids, the ratio of free to conjugated amino acids being 3:2. Histidine constituted 50–66% of the total molarity voided and about 90% of all amino acid nitrogen voided. Arginine constituted 5–6% and glycine ≈15% of the total molarity voided (Briegel, 1986a).

Histidine contains three nitrogen atoms per molecule and therefore is a significant vehicle for nitrogen excretion. Since mosquitoes are incapable of histidine synthesis the excretory histidine cannot be a metabolic product. When fed 4 μl human or guinea-pig blood females of *Ae aegypti* incorporated into their ovaries 14% of the histidine in the human blood and 20% of that in the guinea-pig blood (Briegel, 1985). It seems probable that most or all of the bloodmeal histidine that was not used for oogenesis was not deaminated but eliminated directly.

Small amounts of arginine ($C_6H_{14}N_4O_2$) are also excreted unchanged. The lower concentration of arginine than of histidine in the excreta reflects its lower concentration in mammalian blood and greater incorporation into the ovaries (France and Judson, 1979; Briegel, 1985, 1986a). The conjugation of glycine with toxic compounds is well known, so it may be that the conjugated

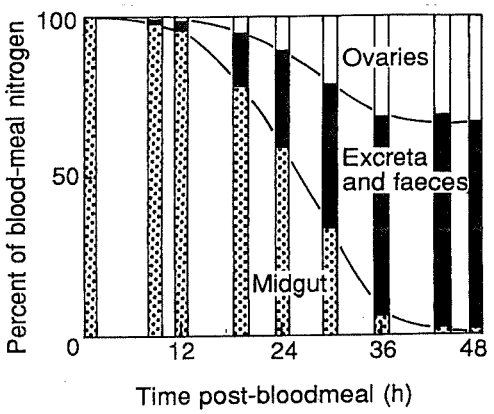

Figure 16.7 Temporal distribution of blood-meal nitrogen during an ovarian cycle between the midgut, excreta + faeces, and ovaries of females of *Aedes aegypti* given 4 μl rat blood by enema. (From Briegel (1986a). Copyright (1986), Pergamon Press plc. Reprinted with permission.)

Table 16.7 Distribution of blood-meal nitrogen between the ovaries and various excretory and faecal constituents 70 h after *Aedes aegypti*, which had either been denied water or given access to water, were fed 3 μl rat blood by enema. When water was provided some ammonia was lost and the recovery of nitrogen was incomplete. Faecal proteins were hydrolysed before assay and are recorded as amino acids. (From Briegel, 1986a.)

	Denied water				*Provided with water*			
	μg N/♀	%N		nMol/♀	μg N/♀	%N		nMol/♀
Blood meal	85.1	100			85.1	100		
Ovaries	36.3	43			36.7	43		
Excreta + faeces	50.5	59			38.6	44		
Uric acid	22.4	26		400	9.1	11		160
Urea	7.1	8		250	13.5	16		480
Ammonium ion	9.7	11	60%	690	6.6	8	43%	468
Amino acids	10.3*	12		340	5.4†	6		220
Haematin	2.1	3		38	2.2	3		38

* 68% of amino N was in histidine (166 nMol/female).
† 43% of amino N was in histidine (54 nMol/female).

glycine found in the excreta was part of a detoxification product.

The faeces contained haematin, digestive enzymes and the remnants of the peritrophic membrane. Upon the digestion of haemoglobin haem is oxidized to haematin and is not absorbed, consequently this source of nitrogen is eliminated in the faeces. The output of faecal haematin showed an exact stoichiometric relationship with the haemoglobin input, four moles of haematin being produced for each mole of dietary haemoglobin, irrespective of blood volume or host species. Small amounts of protease were discharged throughout the period of digestion, but most was voided with the haematin. The digestive enzymes that were discharged included trypsin, aminopeptidase and carboxypeptidase, still in their active forms. The total of this proteolytic activity was over half that in the midgut at the time of maximum activity (Briegel, 1975, 1986a; Graf and Briegel, 1982).

Quantitative analyses made during an ovarian cycle of *Ae aegypti* revealed that the total nitrogen content of the pooled excreta, faeces and mature ovaries always equalled the blood-meal nitrogen intake; consequently the mean nitrogen content of the female body before the blood meal and after oviposition were identical. In one experiment, with females fed 3 μl rat blood, the total nitrogen content per female was 60.7 ± 3.8 μg before feeding and 60.8 ± 3.33 μg after oviposition. Of the 82.8 ± 3.3 μg nitrogen ingested, 29.7 ± 6.7 μg was utilized for yolk protein synthesis and 48.2 ± 4.1 μg was excreted. Clearly the ingested nitrogen was either utilized for oogenesis or excreted.

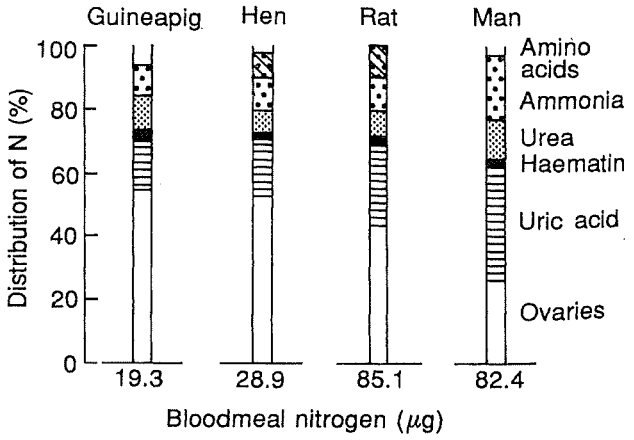

Figure 16.8 Budget of dietary nitrogen in *Aedes aegypti* at the end of an ovarian cycle. Females were given 1 μl guinea-pig or hen blood or 3 μl rat or human blood, and the percentage incorporation of nitrogen into the mature ovaries and the excretory and faecal constituents was determined. The examples were chosen on the basis of maximum nitrogen recovery (⩽ 93%). Except with rat blood, excreted amino acids were not accounted for. (From Briegel (1986a). Copyright (1986), Pergamon Press plc. Reprinted with permission.)

The extent of nitrogenous excreta produced by *Ae aegypti* from the blood of four different hosts reflected the dynamics of yolk synthesis. At one extreme only 26% of the nitrogen in human blood, which is characterized by its low isoleucine content, was incorporated into the ovaries while 70% appeared in nitrogenous metabolites. At the other extreme, 55% of the nitrogen in guinea-pig blood was incorporated into the ovaries and 35% appeared as nitrogenous metabolites (Figure 16.8) (Briegel, 1986a). Complete nitrogen budgets for *Anopheles albimanus* and *An stephensi* revealed that approximately 80% of blood-meal protein was catabolized and excreted through the three major pathways that terminated in uric acid, urea and ammonia (Briegel, 1990b).

16.6 REGULATION OF EXCRETION AND DEFAECATION AFTER BLOOD FEEDING

Processing of the blood meal includes three phases of elimination of unwanted substances. Immediately after feeding excess water and ions are discharged. Later, as protein digestion starts, nitrogen excretion also starts and excretion peaks when the rate of proteolysis reaches its maximum. Finally, after digestion has been completed residual substances in the midgut are voided. Excretion and defaecation can be distinguished visually because the excreta, coloured white or pale yellow by the uric acid, appear first and the dark brown or black faeces, coloured by haematin, appear later; however, there is some overlap.

Females of *Aedes aegypti* kept at 27 or 28°C discharged excreta gradually from about 12 to 36 h post-bloodmeal, and discharged faeces during a relatively short period between 24 and 48 h post-bloodmeal (Figure 16.9). Occasionally up to 90% of the haematin was voided within a period of 3–4 h, but in most instances the process lasted 8–12 h. Small amounts of proteinase were discharged between 12 and 24 h post-bloodmeal but most was voided during the period of haematin discharge. The time of defaecation varied slightly with size of blood meal. By the time haematin had been voided the alimentary canal was empty. It was extremely rare for oviposition to start before all haematin had been voided from the mid- and hindgut (Briegel, 1975, 1986a; Gillett *et al.*, 1975).

Females of *Ae aegypti* that were decapitated immediately after blood feeding did not undergo vitellogenesis but nevertheless showed a similar temporal pattern of uric acid excretion to control females, but with a lag of four hours or more. The decapitated females excreted additional uric acid which corresponded precisely with the amount of nitrogen usually utilized in the production of eggs (Briegel, 1980b).

Females decapitated 32 h after feeding defaecated normally at 50–60 h whereas females decapitated 28 h after feeding retained most of the haematin in the gut. Briegel (1980b) concluded that possibly, between 28 and 32 h after feeding, nervous stimulation of the alimentary canal controlled subsequent defaecation. The time of defaecation is an inherited character. Strains of *Ae aegypti* could be classified as early, intermediate or late according to whether the time by which 50% of females had discharged haematin (the DT_{50}), was 28–32, 33–39 or >40 h post-bloodmeal. When strains were crossed the linear regression of filial on maternal DT_{50} values confirmed the heritability of the trait. Strains that discharged haematin early also discharged uric acid early (Briegel, 1986b).

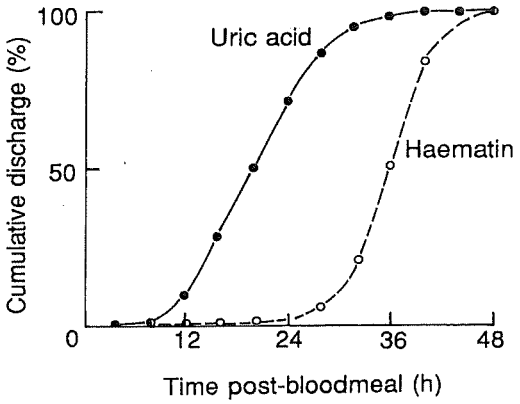

Figure 16.9 Times of discharge of uric acid and haematin by females of *Aedes aegypti* fed 3 µl rat blood and kept at 27°C. Excreta and faeces were collected at 4 h intervals, and the data are plotted as cumulative percentage of total compound discharged. (After Briegel (1986a). Copyright (1986), Pergamon Press plc. Reprinted with permission.)

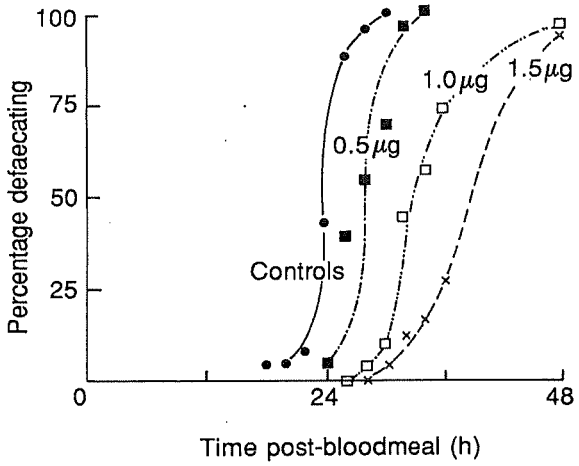

Figure 16.10 Effects of injection with different amounts of 20-hydroxyecdysone upon the time of onset of defaecation in ovariectomized females of *Aedes aegypti*. Control females were injected with saline. The mosquitoes had fed to repletion on human blood and were kept at 28°C. (From Cole and Gillett, 1979.)

Strains of *Ae aegypti* differed by up to six hours in the time by which 50% of females had released ovarian ecdysteroidogenic hormone (OEH). A positive correlation was obtained between that parameter and the time post-bloodmeal by which 50% of females had discharged haematin (Briegel, 1986b), so it seems that the time of OEH release affects the timing of physiological processes that occur up to 36–48 h later.

The involvement of 20-hydroxyecdysone in the control of defaecation has been postulated by Gillett and collaborators, who recorded the time of onset of haematin discharge under different experimental conditions. Females of *Ae aegypti* that took very small blood meals and failed to mature their ovaries defaecated 12 h or more before the controls. Early discharge of the gut contents was also caused by removal of the MNC perikarya or of the ovaries prior to the ingestion of a full blood meal, and by decapitation immediately after blood feeding. All of these operations prevented vitellogenesis. The gut contents of small feeders were not discharged prematurely if 0.1 µg 20-hydroxyecdysone was injected. Similarly the effects of decapitation and ovariectomy could be prevented by injecting 20-hydroxyecdysone 4 h post-bloodmeal. A positive correlation was obtained between the dose of 20-hydroxyecdysone injected into ovariectomized females, within the range 0.5–1.5 µg, and the delay in onset of defaecation (Figure 16.10). It was concluded that the 20-hydroxyecdysone secreted after blood feeding not only stimulates vitellogenesis but also prolongs the period of retention of the blood meal (Gillett *et al.*, 1975; Cole and Gillett, 1978, 1979). Similar results were obtained with *Anopheles freeborni* (Rosenberg, 1980).